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Regular Articles

Monopolar preparation of human lymphocytes for evaluation of the metaphase chromosome alignment

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Abstract. The metaphase alignment of chromosomes to the midzone is indispensable for the faithful segregation of sister chromatids in the process of cell division. In the present study, we describe a new method for the collection of monopolar-arrested human lymphocytes by a combination of monastrol treatment and low speed centrifugation (spin-down). In this preparation, cells with non-aligned chromosomes are easily discernible from those with complete alignment of chromosomes. Immunostaining of spindle and kinetochores is helpful for the accurate judgment whether chromosome alignment is complete or not. In non-immortalized lymphocytes (T-cells) and an immortalized lymphoblastoid (B-cells) cell line derived from control adults, greater than approximately 80% of cells among the metaphase population showed complete alignment of chromosomes by the monopolar/spin-down preparation. In contrast, incomplete alignment was found in 35-55% cells in lymphocytes derived from a Cornelia de Lange syndrome patient with a mutation in *NIPBL* gene. Homologues of *NIPBL* in yeast (Scc2) and *Drosophila* (*Nipped-B*) are required for sister chromatid cohesion during mitosis as a regulator of the binding of cohesin to DNA. Since the importance of chromosome segregation machinery is recently accentuated by the detection of congenital disorders with mutations in genes involved in chromosome assembly and segregation, the monopolar/spin-down preparations using human lymphocytes may be a useful tool for the evaluation of the quality of chromosome dynamics in the clinical context of these disorders.

Keywords: Lymphocytes, Monastrol, Monopolar, Chromosome alignment, Chromosome assembly

Introduction

To study morphological and structural changes of chromosomes in cytogenetics, cells are usually arrested at metaphase with an inhibitor of microtubule polymerization, such as colchicine or colcemid, and then are treated with a hypotonic KCl solution and Carnoy's fixation (methanol : acetic acid = 3 : 1). These treatments are essential for "high-quality chromosome preparations", in which each chromosome is easily identified by morphology and differential staining methods (G-banding, Q-banding, or R-banding). Recently, it has been reported that a number of genes implicated in chromosome assembly and segregation are associated with congenital disorders, for example ICF syndrome (Wijmenga *et al.*, 2000), PCS/MVA syndrome (Hanks *et al.*, 2004; Lampson and Kapoor, 2005; Matsuura *et al.*, 2006), Cornelia de Lange syndrome (Tonkin *et al.*, 2004; Kaur *et al.*, 2005), hereditary microcephaly (Trimborn *et al.*, 2004; Lin *et al.*, 2005; Woods *et al.*,

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2005), and Roberts syndrome (Vega *et al.*, 2005). In ICF syndrome, centromeric instability of chromosomes 1, 9, and 16 is cytogenetically found, and PCS/MVA syndrome shows a typical aberration of chromosome segregation, premature chromatid separation (PCS). However, in other cases, it is difficult to detect morphological aberration of chromosome by routine cytogenetic methods. A possible reason is that microtubule-kinetochore attachment is perturbed by use of colchicine or colcemid (Borisy and Taylor, 1967; Rieder and Palazzo, 1992), indicating that cytogenetic preparation is made under "an abnormal condition" in the viewpoint of chromosome assembly and segregation. Thus, development of preparation method for the study of chromosome assembly and segregation may be helpful in clinical diagnosis of chromosome segregation disorders and further delineation of the cellular defects in these disorders.

In metaphase, chromosomes are aligned to the center of the cell (metaphase plate) prior to the onset of anaphase. Accumulating lines of evidence show that the chromosome alignment is accomplished by an orchestration of many mitotic events, including chromosome assembly and segregation (Vass *et al.*, 2003; Chang *et al.*, 2003; Ono *et al.*, 2004), bipolar formation of mitotic spindle and kinetochores (Levesque and Compton, 2001; Nishihashi *et al.*, 2002), regulation of cell cycle progression dependent on reversible phosphorylation by mitotic kinases (Giet and Glover, 2001; Donaldson *et al.*, 2001; den Elzen and Pines, 2001; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003) and checkpoint activation (Skoufias *et al.*, 2001). Actually, we previously reported that knockdown of the chromosomal protein complexes, condensin I and condensin II, induced severe irregular localization of chromosomes in monastrol-treated HeLa cells (Ono *et al.*, 2004). Accordingly, chromosome alignment may be a useful marker for the diagnosis of human diseases associated with failures in mitosis, especially mechanisms of chromosome assembly and segregation.

In the present study, we applied the monastrol-mediated chromosome alignment method to human lymphocytes. It is known that monastrol, a chemical inhibitor of the mitotic kinesin Eg5, is useful for studying mitotic mechanisms (Mayer *et al.*, 1999; Kapoor *et al.*, 2000). Unlike colchicine nor colcemid, monastrol does not perturb microtubule polymerization, producing a monopolar spindle and a complement of chromosomes aligned on the metaphase plate. A combination of spin-down spreading method and immunolabeling of α-tubulin and kinetochores helps us to examine chromosome alignment in non-adherent cells. We evaluated here the chromosome alignment of human lymphocytes derived from normal individuals and a patient affected with Cornelia de Lange syndrome (CdLS) under monopolar/spin-down condition. Reproducible results suggest that investigation of chromosome alignment in human is effective for diagnosis of congenital anomalies associated with defects of chromosome assembly and segregation.

Materials and methods

Bioethics Approval

Under the committee approval in Aichi Human Service Center, whole blood of a patient affected with CdLS and unaffected individuals were obtained after signed informed consent.

Cornelia de Lange syndrome patient

A female patient was diagnosed with typical clinical features of CdLS one year old. Sequence analysis showed a two-base deletion (5387-5388 del TG) within ORF in *NIPBL* gene, resulting in a frame shift mutation (L1796FfsX1797). Routine chromosome banding analysis of the patient revealed a normal karyotype (2n=46, XX).

EBV infection for establishment of lymphoblastoid cell line (LCL)

After informed consent was obtained, the lymphocytes were isolated from peripheral bloods of a normal adult male (28 years old) and a female patient affected with CdLS (one year old) using Lymphoprep according to the manufacturer's instructions (Nycomed Pharma). The isolated cells were washed with 0.9% NaCl, and then infected with EBV (Epstein-Barr virus) in RPMI 1640 culture medium (Gibco) at 37°C for one hour (Neitzel, 1986). The EBV was prepared from cultured B95-8 cells (JCRB9123) provided by Human Science Research Resources Bank (HSRRB), Japan. After the infection, the medium containing EBV was withdrawn by centrifugation, and the cells were suspended in 0.5 ml of RPMI supplemented with 20% FBS (Sanko Junyaku, Japan) and 2 μg/ml final concentration of the immunosuppressivum Cyclosporin A (CSA: Sandimmun/Novartis) (Ikeuchi and Yamamoto, 1987). For 2 weeks, the culture was performed using 5 ml tubes with round bottom (FALCON; 2058) to ensure high density of the EBV-infected cells. Half of the culture medium (0.25 ml) was changed every 4 days. Then, the EBV-infected cells were subcultured into 24-well dishes with RPMI 1640 containing 20% FBS. For maintaining exponential growth, the cells were subcultured every 3 or 4 days at a split ratio of 1:1 with fresh medium. Establishment of LCL was confirmed by re-culturing of stock cells stored in liquid nitrogen and continuous culture for 3 months.

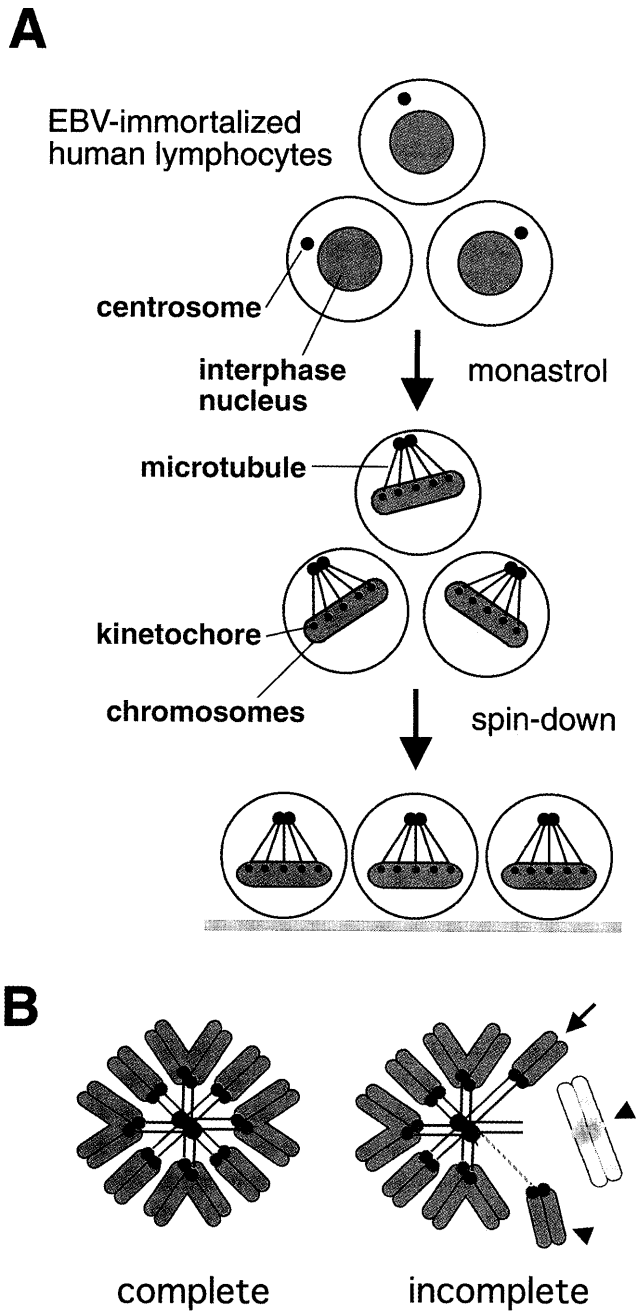

Figure 1. (A) Principle of monopolar/spin-down preparation for human lymphocytes. First, cells were arrested in monopolar metaphase by the inhibition of centrosome separation using monastrol. Second, the cells were spun-down to coverslips for microscopic observation of monopolar spindle and aligned chromosomes. (B) Model for chromosome alignment. Monastral causes monoastral spindles with a rosette-like microtubule array surrounded by a ring of chromosomes. Cells with a continuous ring of chromosomes are classified as "complete alignment", whereas cells with one or more chromosome distant from the alignment ring are categorized as "incomplete alignment". The non-aligned chromosomes are considered to result from defects in chromosome assembly (arrowhead/metacentric chromosome in figure), spindle assembly (arrowhead/acrocentric chromosome in figure), or due to delay of chromosome alignment in the normal process (arrow/acrocentric chromosome). See text in detail.

Short-term culture of lymphocytes

We also isolated lymphocytes from peripheral bloods of two normal adult females (both 20 years old) and the Cornelia de Lange syndrome for a short-term culture using non-immortalized lymphocytes (T-cells). The isolated lymphocytes were stimulated with PHA (Phytohemagglutinin-M ; Gibco). After 68-70 hours culture, the cells were processed with monastrol.

Monopolar alignment and spin-down of the chromosomes

Outline of the protocol for monopolar/spin-down preparations for lymphocytes is shown in Fig. 1. To align chromosomes on a monopolar spindle, cells were treated with 100 μM monastrol (Calbiochem) in the culture medium for 5 hours (Leizerman *et al.*, 2004 ; Hauf *et al.*, 2003 ; Ono *et al.*, 2004). During the period of monastrol treatment, the cells are arrested with monopolar spindles by inhibition of the motility of the mitotic kinesin Eg5, preventing the separation of duplicated centrosomes (Mayer *et al.*, 1999 ; Kapoor *et al.*, 2000). But, the polymerization of microtubules is not inhibited with monastrol, so that the cells display a cone-like mitotic spindle. After alignment, spindle force and each kinetochore's force reach a situation of equilibrium. In HeLa cells on coverslips, the chromosomes line up in the bottom under monopolar spindle, instead of metaphase plate, because chromosomes are heavier than centrosomes and microtubules. Therefore, the single pole in HeLa cells is located at the center and chromosomes are aligned in a rosette-like distribution (Hauf *et al.*, 2003 ; Ono *et al.*, 2004). Although we found that monastrol is effective in suspended lymphocytes and EBV-immortalized LCLs, the polarity of suspended lymphocytes with monopolar spindle was compromised through KCl-hypotonic treatment, Carnoy's fixation, and spreading procedure (data not shown).

Then, lymphocytes treated with monastrol were centrifuged at low speed (800 rpm) for 2 minutes onto poly-L-lysine-coated coverslips using Auto-Smear CF-12D (Sakura Finetek, Japan), to maintain the polarity of the cells with monopolar spindle (Fig. 1).

Immunofluorescent staining of monastrol-arrested cells

After spreading of the cells on coverslips by centrifugation (spin-down), monastrol-arrested cells were immediately fixed with 2% fresh paraformaldehyde in PBS (pH 7.4) at room temperature for 15 minutes, followed by permeabilization with 0.5% Triton X-100 in PBS for 5 minutes (Losada *et al.*, 1998 ; Ono *et al.*, 2003). The cells were subjected to immunofluorescence analysis according to Ono *et al.* (2004) with slight modifications. In brief, for double immunolabeling of kinetochores and microtubules, we used human autoim-

mune anti-centromere antibody (ACA/CREST serum) (Muro *et al.*, 1990) and monoclonal anti-α-tubulin (Clone DM1A, Sigma), respectively. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-human IgG were used as secondary antibodies (Molecular probes). After counterstaining with DAPI (Roche), the coverslips were placed on slides, mounted with Vectershield (Vector), and examined with a BX60 fluorescence microscope using a combination of filter cubes, U-MNIBA for Alexa Fluor 488, U-MWIG for Alexa 594 and U-MWU for DAPI (Olympus). Grayscale images were pseudocolored and merged using Adobe Photoshop.

Cytogenetic chromosome spread

In order to evaluate the occurrence of premature chromatid separation (PCS), LCLs were used for routine cytogenetic preparation. Cells were treated with 0.2 μg/ml colchicine for 1 hour at 37°C, resuspended in a hypotonic 0.075M KCl solution for 12 or 20 min at 37°C, and then fixed with Carnoy's solution (Methanol : acetic acid = 3 : 1). Chromosome spreading was performed under a humid condition using water bath. The preparations were stained with Giemsa solution (MERCK).

Results and discussion

In the present study, we developed a method for spin-down chromosome preparations with monopolar spindle in human lymphocytes. Since blood samples are used for routine chromosomal diagnosis in congenital anomalies and many LCLs derived from congenital disorders have been stocked so far, our method could be widely applied in the field. To test the usability of monopolar/spin-down preparation as a method for the evaluation of chromosome alignment, we carried out three independent experiments in a control LCL (Table 1) and examined non-immortalized lymphocytes (Table 2). Similar examination was done in cells derived from *NIPBL* mutation-positive CdLS patient (Table 3). In these preparations three chromosomal phenotypes, com-

Table 1. Evaluation of chromosome alignment in monastrol-arrested human lymphoblastoid cells.

Phenotype*	Number of cells observed (%)		
	LCL-1	LCL-2	LCL-3
Complete alignment	149 (87.6)	161 (81.0)	170 (85.9)
Incomplete alignment	20 (11.8)	38 (19.0)	26 (13.1)
Bipolar spindle	1 (0.6)	0 (0)	2 (1.0)
Total	170	199	198

EBV-immortalized human lymphoblastoid cell line (LCL) derived from a normal male was treated with monastrol for 5 hours, followed by the spin-down preparation, and then subjected to immunostaining (IF) for the evaluation of chromosome alignment. This table shows the results of three independent experiments. *Phenotypes are described in the text and Fig. 1.

Table 2. Evaluation of chromosome alignment in monastrol-arrested human lymphocytes (T-cells) stimulated with PHA.

Phenotype*	Number of cells observed (%)	
	Control-1	Control-2
Complete alignment	262 (94.2)	232 (96.3)
Incomplete alignment	16 (5.8)	9 (3.7)
Bipolar spindle	0 (0)	0 (0)
Total	278	241

Control lymphocytes (T-cells) derived from two females were treated with monastrol for 5 hours, followed by the spin-down preparation, and then subjected to immunostaining (IF) for the evaluation of chromosome alignment. *Phenotypes are described in the text and Fig. 1.

Table 3. Evaluation of chromosome alignment in monastrol-arrested lymphoblastoid cells and PHA-stimulated T-cells derived from a patient affected with Cornelia de Lange syndrome (CdLS).

Phenotype*	Number of cells observed (%)			
	LCL-1	LCL-2	LCL-3	PHA
Complete alignment	173 (62.7)	222 (67.1)	130 (52.6)	81 (42.6)
Incomplete alignment	97 (35.1)	108 (32.6)	111 (45.0)	104 (55.8)
Bipolar spindle	6 (2.2)	1 (0.3)	6 (2.4)	3 (1.6)
Total	276	331	247	188

EBV-immortalized human lymphoblastoid cell lines (LCL) derived from a patient with a mutation in *NIPBL*, homologous to Scc2, was treated with monastrol for 5 hours, followed by the spin-down preparation, and then subjected to immunostaining (IF) for the evaluation of chromosome alignment. This table shows the results of three independent experiments. *Phenotypes are described in the text and Fig. 1.

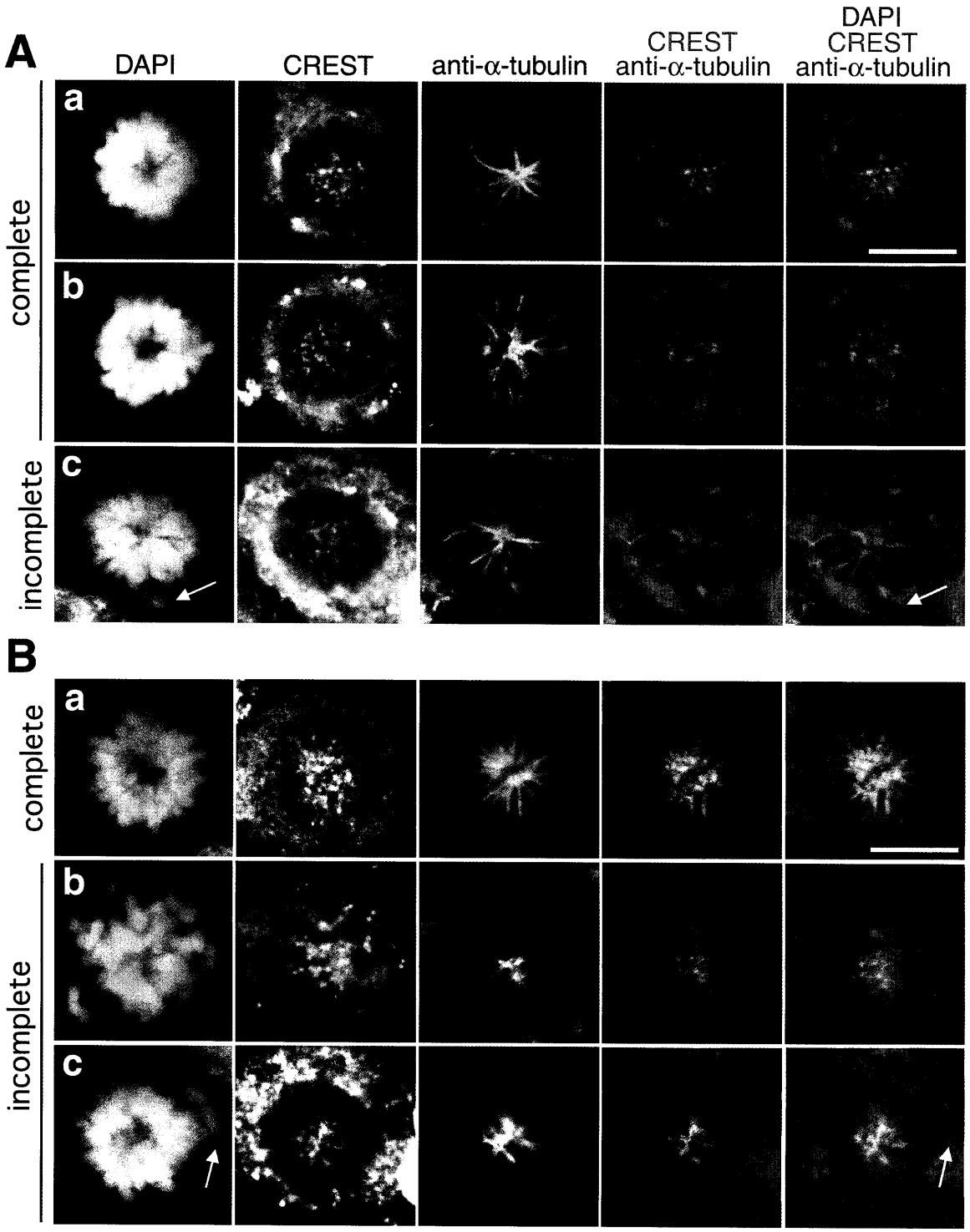

Figure 2. Chromosome alignment of human lymphoblastoid cells arrested by monastrol. EBV-immortalized human lymphoblastoid cell lines (LCL) were arrested using monastrol for 5 hours. After spin-down onto coverslips, they were labeled with anti-α-tubulin and human CREST serum, and stained with DAPI. (A) A LCL derived from an unaffected individual. Two examples of complete alignment (a, b) and one of incomplete alignment (c) are shown. The arrow indicates the non-aligned chromosome distant from a ring of aligned chromosomes. (B) A LCL derived from a patient with Cornelia de Lange syndrome (CdLS). Complete alignment (a) and incomplete alignment (b, c). Bar=10 μm. CREST serum used here includes antibodies of centromeric proteins, CENP-A (IgG and IgM classes), CENP-B (IgG class) CENP-C (IgG and IgM classes), as previously reported (Muro, *et al.*, 1990). These proteins may be located not only chromosomal centromeres but also cytoplasm, resulting in "outside" CREST-positive red staining besides the CREST-positive red dots on chromosomes.

Table 4. Evaluation of chromosome alignment in monastrol-arrested HeLa cells.

Phenotype*	Number of cells observed (%)
Complete alignment	178 (76.7)
Incomplete alignment	18 (7.8)
Bipolar spindle	36 (15.5)
Total	232

HeLa cells cultured on the coverslips were treated with monastrol for 5 hours, and then fixed in culture well without spin-down.
*Phenotypes are described in the text and Fig. 1.

plete alignment, incomplete alignment, and bipolar spindle, were observed (Fig. 2 and Tables 1-3). As shown in Fig. 2, immunostaining with anti-α-tubulin and CREST serum, that delineate spindle formation and localization of kinetochores, respectively, is helpful for the accurate judgment whether chromosome alignment is complete or not. Complete alignment chromosomes display a rosette-like spindle where chromosomes are uniformly radiated from a single pole. Under the present condition in monastrol treatment, greater than 80% of cells showed a phenotype of complete alignment in a control LCL and greater than 90% of non-immortalized lymphocytes showed complete. Cells with bipolar spindles, which may have escaped monastrol inhibition, appeared rarely in controls (Tables 1 and 2). These results indicate that monastrol is effective in human lymphocytes and EBV-immortalized LCLs as in

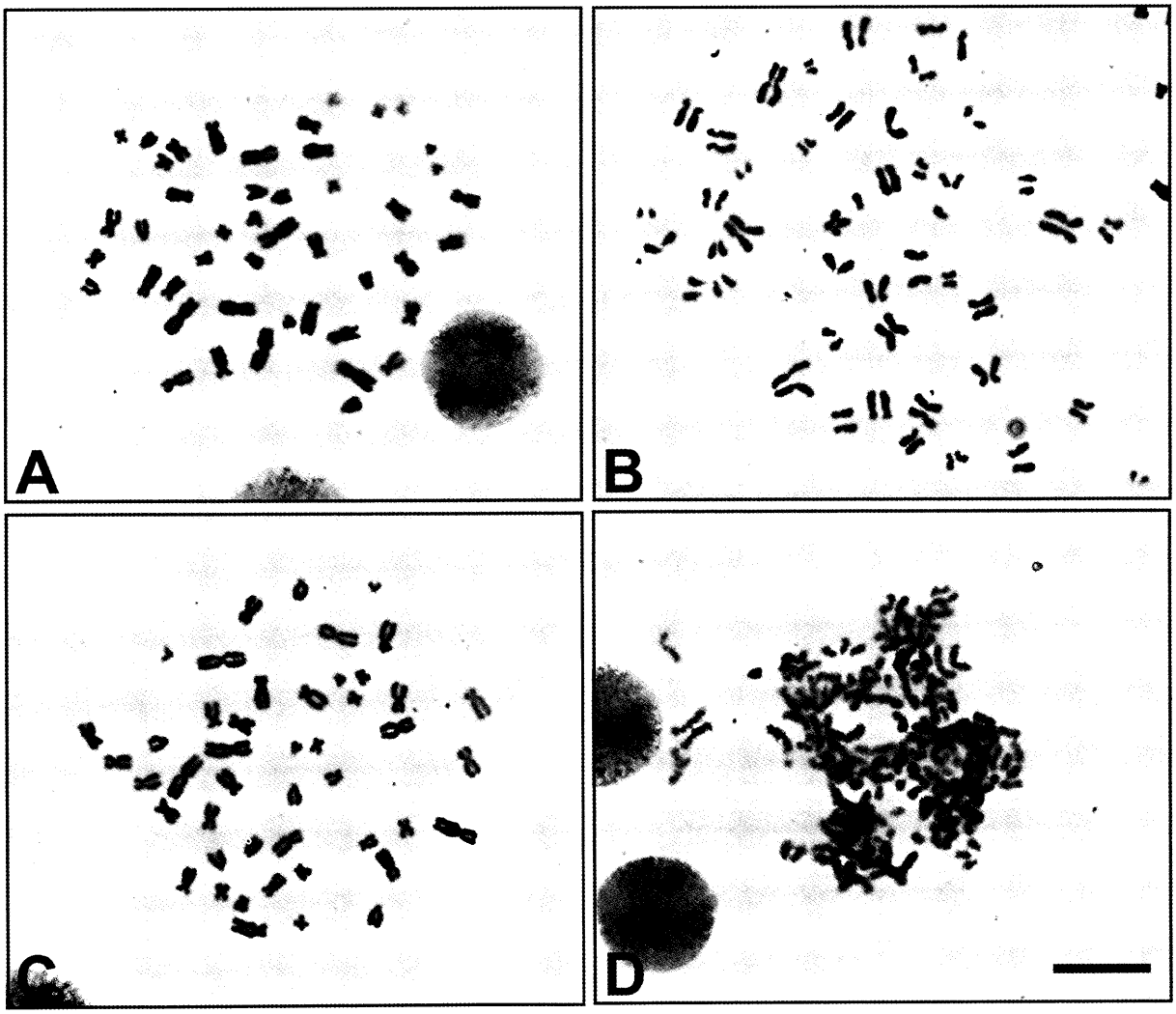

Figure 3. Metaphase spreads in two LCLs derived from an unaffected control (A and B) and a CdLS patient (C and D). Cells were treated with 0.075M KCl followed by Carnoy's solution. Incidence of premature chromatid separation (PCS) positive metaphases (B and D) was lower than 1% in both control and affected LCLs. Frequency of each phenotype is shown in Table 5. Bar=10 μm.

Table 5. Incident of premature chromatid separation (PCS) in colchicine-arrested metaphases of lymphoblastoid cells from a patient affected with Cornelia de Lange syndrome (CdLS) and control.

Phenotype	Number of cells (%)			
	Control LCL		CdLS LCL	
	12 min KCl	20 min KCl	12 min KCL	20 min KCl
Normal metaphase	144 (99.3)	194 (99.5)	153 (98.7)	266 (99.3)
PCS	1 (0.7)	1 (0.5)	2 (1.3)	2 (0.7)
Total	145	195	155	268

EBV-immortalized human lymphoblastoid cell lines (LCLs) derived from a patient with a mutation in *NIPBL* and normal male were treated with colchicine for 1 hours, followed by hypotonic treatment of 12 min or 20 min with KCl. Then, the cells were fixed with methanol : acetic acid (3 : 1). The slides were stained with 2% Giemsa solution.

other types of mammalian cells (Mayer *et al.*, 1999; Kapoor *et al.*, 2000; Ono *et al.*, 2004: Leizerman *et al.*, 2004).

In cells classified as "incomplete alignment", chromosomes were not continuously aligned and often some chromosomes were found distant from the radiated aligned complement (Fig. 1). Frequency of cells with incomplete alignment varied from 11.8% to 19.0% in a control LCL (Table 1), and 3.7 and 5.8% in two control individuals (Table 2), whereas that varied from 32.6% to 45.0% in LCL and 55.8% in non-immortalized lymphocytes from the present CdLS patient (Table 3). In monopolar cells, complete alignment corresponds to "metaphase", while incomplete alignment is considered to correspond to "prometaphase". In cytogenetic preparation, frequency of cells with prometaphase chromosomes depends on condition of colchicines such as time and concentration. Without spin-down, incomplete alignment was also found in 7.8% of monopolar HeLa cells (Table 4). Thus, the low incidence of incomplete alignment in the control lymphocytes represent probably cells that enter the mitosis at a late period of monastrol treatment and had not finished chromosome alignment to metaphase plate, termed "temporal delay" of chromosome alignment. On the other hand, we found that frequency of incomplete in control LCL is little higher than that in non-immortalized lymphocytes. This increase of incomplete in chromosome alignment may be involved in establishment and/or long-term culture of lymphocytes. Although it is unclear whether EBV-immortalization could influence chromosome alignment, it is known that infection of HIV in lymphocytes causes failure of chromosome assembly and segregation (Saadat *et al.*, 1998; Shimura *et al.*, 2005).

A high incidence of incomplete alignment in CdLS examined here may indicate "constitutive" delay or failure of chromosome alignment, because *NIPBL* gene in the present patient possessed a frame shift mutation. Homologues of *NIPBL*, Scc2 (yeast) and *Nipped-B* (*Drosophila*) are essential for sister chromatid cohesion during mitosis as a regulator of the binding of cohesin to duplicated DNA (Tonkin *et al.*, 2004; Kaur *et al.*, 2005). It is, therefore, most likely that CdLS cells have a defect on chromosome assembly and segregation. Our case, however, did not show any segregation defect of chromosomes, such as premature chromatid separation in cytogenetic preparation (PCS) (Fig. 3, Table 5), different from results of Kaur *et al.* (2005). Thus, our results suggest that the primary chromosomal defect of CdLS with *NIPBL* mutation is delay or failure of chromosome alignment rather than the numerical aberration of chromosomes. In general, it is speculated that the delay or failure of chromosome alignment might cause apoptotic cell death and shortage of cells necessary for the development. Clearly, further works are required to test this hypothesis.

As described in the introduction, it has been recently shown that some human genetic diseases are tightly associated with defects in chromosome assembly, chromosome segregation, or chromatin modification. Some but not all of them accompanied by detectable defects in chromosomal morphology or behavior under a light microscope. Intriguingly, most of these diseases are associated with congenital defects in brain size or functions. It will be of great interest to determine the precise relationship between basic chromosome functions and brain development. The present results demonstrated that the defect of chromosome alignment is associated with *NIPBL*-mutation positive CdLS. Furthermore, our monopolar/spin-down method may be an useful approach for congenital anomalies associated with defects of chromosome assembly and segregation. In the future, analyses using monastrol, combined with the identification of specific chromosomes or subchromosomal regions by FISH, may result in a better understanding of the chromosome dynamics during mitosis and the context of human diseases associated with chromosome assembly and segregation.

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